NGS Library Protocols

I. Total DNA from leaf tissue of infected cassava

- Starting material for this protocol is total DNA extracted from leaf tissue using either Qiagen Plant DNA or ThermoFisher Mag Max kits.
- Specifically, this protocol was tested using infected cassava material with the infecting viral DNA as the target DNA for the library construction.

Rolling Circle Amplification using EquiPhi29 (Set up all reactions on ice)

- Anneal random hexamer primers to the DNA.
  - Make a preliminary master mix of 10X reaction buffer, exo-resistant random primers and water. Add 3 µl of this master mix to 2 µl of total DNA.

1) A 1x Reaction consists of the following:

Component	Volume	Final Concentration
gDNA	2.0 µl (regardless of concentration)	--
10X reaction buffer	0.5 µl	1x
Exo-resistant random primers (500 µM) (#SO181ThermoFisher)	1.0µl	100µM
Nuclease free water	1.5µl	--
Total volume	5.0 µl	

2) Heat at 95°C for 3 minutes and immediately place on ice for an additional 3-5 minutes to allow annealing of the primers to the denatured DNA.

- Amplify circular DNA
  - Make a second master mix of 10X reaction buffer, DTT, dNTPs, EquiPhi29 polymerase, pyrophosphatase and water. Add 15µl of this master mix to the 5µl DNA/primer step from above.

3) A 1x Reaction consists of the following in the table below:

Component	Volume	Final Concentration
Denatured/primer annealed DNA	5.0µl	--
10x Reaction Buffer for EquiPhi29	1.5µl	1x
DTT (100mM)	0.2µl	1mM
dNTP mix 10mM each	2.0µl	1mM each
EquiPhi29 DNA polymerase (10U/µl) (#A39390)	1.0µl	10U
Pyrophosphatase (0.1U/µl) (#EF0221)	1.0µl	0.1U
Nuclease-free water (up to 20µl)	9.3µl	
Total:	20µl	

4) Incubate at 40°C for 2 hours.
5) Heat inactivate the enzymes at 65°C for 10 minutes.

Fill-in single stranded DNA gaps in the RCA product (Set up all reactions on ice)

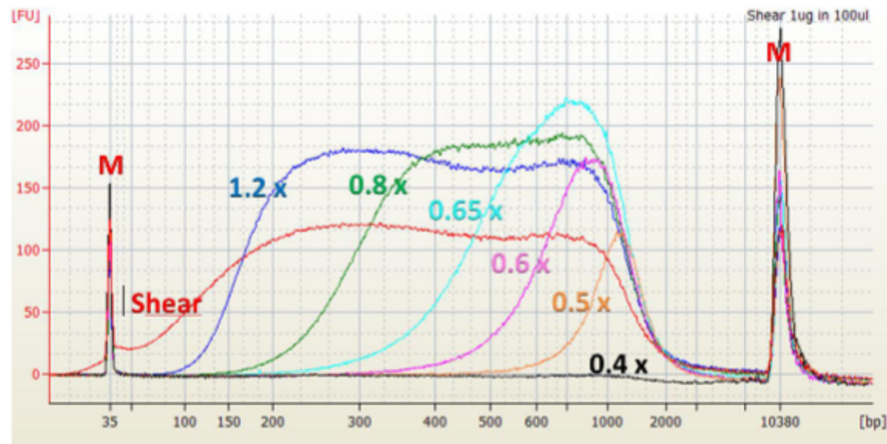
- Make a master mix of nuclease free water, 10X NEB buffer #2, dNTPs, Klenow fragment, and T4 DNA polymerase.
- A 1x reaction consists of the following:

Component	Volume	Final concentration
Nuclease-free water	22.5µl	--
10x NEB buffer #2	5.0µl	1X
dNTP (10mM)	0.5µl	0.1mM
Klenow (Lg. subunit DNA Pol I	1.0µl	5U
T4 DNA polymerase	1.0µl	3U
DNA from RCA reaction	20µl	--
Total volume	50µl	

1) Pipette master mix up and down to mix.
2) Spin down briefly.
3) Add 30µl of the master mix to each RCA reaction for a final reaction volume of 50µl .
4) Spin down briefly.
5) Incubate tubes under the following parameters:
   - 25°C for 1 hour
   - 75°C for 20 minutes

SPRIselect magnetic bead cleanup (Perform steps at room temperature)

Figure 1 Agilent High Sensitivity DNA chip Electropherogram.



M = upper and lower markers for High Sensitivity DNA chip

Shear = 1 µL of 20 ng/µL input control sample in water

1.2x to 0.4x = 1 µL of shear, size selected with given ratio of **SPRIselect** volume to sample volume.

In order to purify the DNA from the salts and enzymes from the previous reactions and retain as much DNA as possible, a 1.2x bead volume to sample volume ratio is used for a Left Side size selection. See Figure 1 above. The SPRI beads at this point are being used as a purification step, not a size selection step.

- Vortex/shake SPRIselect magnetic beads (stored at room temperature) until beads are in homogenous suspension.
- Periodically shake or vortex the bottle containing the beads to maintain the beads in suspension during pipetting of large numbers of samples.
- Make fresh 80% ethanol.
- Keep lids closed on tubes will not pipetting, especially during resuspension steps. Open each tube individually to add or remove liquid.

1) Briefly spin down RCA/fill-in product.
2) Check to make sure that each product volume is 50µl. Add water up to 50µl if it is less or remove liquid to decrease total to 50µl.
3) Add 60µl (1.2x) of room temperature SPRIselect beads in suspension to the RCA/fill-in product.
4) Pipette up and down 10 times to mix.
5) Briefly spin down tube (just enough to get liquid off sides of tubes, NOT long enough to pellet beads, the beads need to stay in suspension).
6) Incubate at room temperature for 5 minutes.
7) Place tubes on magnetic rack for 5 minutes or until liquid is clear.

8) Carefully remove and discard liquid without disturbing pellet.
9) Add 200µl of 80% ethanol to the beads while on the magnet and incubate for 30 seconds
10) Carefully remove and discard liquid without disturbing pellet.
11) Again, add 200µl of 80% ethanol to the beads while on the magnet and incubate for 30 seconds.
12) Carefully remove and discard liquid without disturbing pellet.
13) Remove tubes from the magnetic rack and briefly spin down the tubes.
14) Place the tubes back on the magnetic rack and wait for the pellet to form on the tube wall.
15) Use a P10 (10µl) tip to remove residual ethanol.
16) Leave lids open and air dry the pellets on the rack for 5 minutes. Do not over dry the pellet as this will result in a loss of DNA.
17) Close lids and remove the tubes from the rack
18) Immediately add 17µl nuclease-free water to each tube, then close lids. Then pipette up and down to suspend the pellet in each tube.
19) Incubate the tubes for five minutes at room temperature.
20) Briefly spin the tubes and place tubes on the magnetic rack for 5 minutes or until the liquid is clear.
21) Without disturbing the pellet, transfer 15µl of the liquid to a new PCR tube.

- Quantify the purified DNA above using a Qubit assay in order to determine the dilution necessary for 1ng total DNA as input for the Nextera XT DNA library kit.

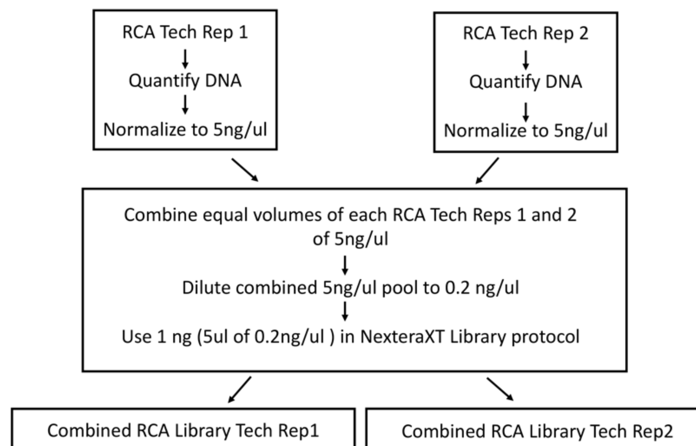
Qubit quantification (Perform steps at room temperature)

- Make a Qubit Working Solution using either Qubit Broad Range (BR) or High Sensitivity (HR) dsDNA Buffer depending on the suspected concentration range.
    a. BR has a detection range between 2ng to 1,000ng.
    b. HS has a detection range between 0.2ng to 100ng.
- Calculate the number of samples to be quantified plus two standards and multiply that number by 200 to determine the volume in ul of the final Qubit working solution.
- Prepare the Qubit working solution by diluting the Qubit BR or HS dsDNA Reagent (dye) 1:200 in Qubit Buffer.
    a. Example: If there are 8 samples plus 2 standards (10 total tubes), then the final working solution volume will be 2,000µl. Add 10µl of Reagent (dye) to 1,990µl of buffer.
- Vortex the Qubit working solution (buffer and dye).
- Transfer 190µl of the working solution (with dye) into two Qubit Assay Tubes for the two standards and 198µl of the working solution into the Qubit Assay Tubes for the unknown samples.
- Add 10µl of standard #1 to one tube of 190µl working solution (final volume is 200µl) and 10µl of standard #2 into the other tube of 190µl working solution.
- Add 2µl of each unknown sample to its own Qubit Assay tube of 198µl working solution (final volume is 200µl).
- Vortex all tubes for 3 seconds.

- Incubate all tubes at room temperature for 2 minutes.
- On the Qubit 3.0 Fluorometer, on the Home screen:
    a. Choose dsDNA
    b. Choose either Broad Range or High Sensitivity
    c. Choose Read Standards
        i. Put standard #1 in the fluorometer and close the lid and press read.
        ii. Repeat for standard #2.
    d. Choose Read Samples
        i. Choose the amount of sample (2µl) and the metrics (ng/µl).
        ii. Put your <u>last</u> sample in the fluorometer and close the lid and press read
            1. NOTE: Start with the last sample and go in order backwards. The first sample will be read last. For example if you have samples labeled 1 through 10. Start with tube 10 and go in order backwards until tube 1 is read last. This is because the fluorometer outputs the data in the opposite order in which the samples were read. In the example, the data output will read in order from tube 1 through tube 10.
    e. Insert flash drive.
    f. Choose Data.
    g. Select most recent set of samples at the top of the list.
    h. Choose Export.

Dilution of Input samples for Nextera XT kit (Carry out on ice)

- **The following steps reflect that two technical replicates of RCA were performed, DNA concentrations of those technical replicates were normalized, the normalized technical replicates were combined, and then two libraries were made from the normalized, combined RCA technical replicates.**



1) **Normalize the DNA concentrations of each of the technical replicates to 5ng/µl.**
    a) Based on the concentration determined by the Qubit assay, dilute each RCA/Fill-in/Cleanup sample to 5ng/µl in nuclease-free water.

b) (Desired concentration of sample) x (Desired final volume)/Concentration of sample = Volume of concentrated sample needed.
c) (Desired final volume)-(Volume of concentrated sample needed)=Volume of nuclease free water needed.

2) Combine equal volumes of each of the two 5ng/µl diluted RCA technical replicates into one combined pool. Usually, 2µl of each technical replicate into a 4µl total pooled volume.
3) Dilute each RCA technical replicate pool (4µl) further to 0.2ng/µl in nuclease-free water
   a) (Desired concentration of sample) x (Desired final volume)/(Concentration of sample)= Volume of concentrated sample needed.
   b) (Desired final volume)-(Volume of concentrated sample needed)=Volume of nuclease free water needed.
4) In each of 2 PCR tubes, use 5ul of 0.2ng/µl RCA combined technical replicate pool (for a total of 1ng DNA) for use at step 3 of the Tagmentation phase of the Nextera XT DNA library kit protocol (see below). Two library technical replicates are made from the pooled RCA technical replicates.

Illumina Nextera XT Library Kit Protocol (Cat # FC-131-1096) (Carry out on ice)

- Thaw all reagents for the Illumina Nextera XT DNA library kit on ice and do not vortex.
- Invert reagents 5-6 times and briefly spin down.

Tagmentation of DNA

- **Enzymatic process that fragments and tags gDNA with adapter sequences**

1) Add the following reagents to a PCR tube in the following order:
2) Add 10µl of Tagmentation DNA Buffer (TD).
3) Add 5µl of 1ng total DNA from Step 4 of "Dilution of Input samples for Nextera XT kit."
4) Pipette up and down 10 times.
5) Briefly spin down.
6) Add 5µl of Amplicon Tagment Mix (ATM).
7) Pipette up and down 10 times.
8) Briefly spin down.
9) Put tubes on thermocycler and run TAG program below.

TAG program:

- Set preheat lid to 100°C
- Set volume to 50µl
- 55°C for 5 minutes
- Hold at 10°C

10) When the program reaches 10°C, ***immediately*** add the NT Buffer.
11) Add 5µl of Neutralize Tagment Buffer (NT) to each tube.
12) Pipette up and down 10 times to mix.
13) Briefly spin down.

14) Incubate at room temperature for 5 minutes.

Amplification of tagmented DNAs

- **The process adds the unique dual index adapters and amplifies the tagmented DNA.**

1) To each tube, add 10µl of IDT Unique Dual pre-paired i7 and i5 adapters (Cat #'s 20027213, 20027214, 20027215, & 20027216)
2) Record which adapters correspond to which libraries.
3) Add 15µl of the Nextera PCR master mix (NPM).
4) Pipette up and down 10 times to mix.
5) Briefly spin down.
6) Put tubes on thermocycler and run the NXT PCR program.

NXT PCR program:

- Set preheat lid to 100°C
- Set volume to 50µl
- 72°C for 3 minutes
- 95°C for 30 seconds
- 12 Cycles of:
    - 95°C for 10 seconds
    - 55°C for 30 seconds
    - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

SPRIselect magnetic bead size selection (Perform steps at room temperature)

In order to remove any adapter dimers and small DNA fragments after the PCR amplification step, a 0.8x bead volume to sample volume ratio is used for a Left Side size selection.

- Follow the SPRIselect magnetic bead cleanup protocol above but modify the following steps:
    1) Add 0.8x beads to sample volume (40µl of SPRIselect beads).
    2) Elute the DNA by adding 52µl nuclease-free water.
    3) Without disturbing the pellet, transfer 50µl of water and DNA to a new tube.

- Quantify the DNA libraries above using Qubit dsDNA HS assays and determine the library quality and average fragment size in base pairs using a Bioanalyzer chip.

Qubit quantification (Perform steps at room temperature)

- For DNA libraries, make a Qubit Working Solution always using High Sensitivity (HR) dsDNA Buffer according to the protocol for Qubit quantification above.

Bioanalyzer Chip Electrophoresis (Perform steps at room temperature)

- In order to check the library quality and average fragment size in base pairs, follow the protocol below for Agilent High Sensitivity dsDNA chip electrophoresis

Running a chip

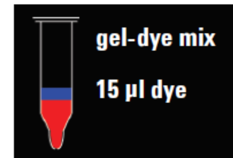
Preparing the Gel-Dye Mix

1 Allow High Sensitivity DNA dye concentrate (blue ●) and High Sensitivity DNA gel matrix (red ●) to equilibrate to room temperature for 30 min.

2 Add 15 µl of High Sensitivity DNA dye concentrate (blue ●) to a High Sensitivity DNA gel matrix vial (red ●).

3 Vortex solution well and spin down. Transfer to spin filter.

4 Centrifuge at 2240 g ± 20 % for 10 min. Protect solution from light. Store at 4 °C.



Loading the Gel-Dye Mix

1 Allow the gel-dye mix equilibrate to room temperature for 30 min before use.

2 Put a new High Sensitivity DNA chip on the chip priming station.

3 Pipette 9.0 µl of gel-dye mix in the well marked G.

4 Make sure that the plunger is positioned at 1 ml and then close the chip priming station.

5 Press plunger until it is held by the clip.

6 Wait for exactly 60 s then release clip.

7 Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.

8 Open the chip priming station and pipette 9.0 µl of gel-dye mix in the wells marked G.

Loading the Marker

1 Pipette 5 µl of marker (green ●) in all sample and ladder wells. Do not leave any wells empty.

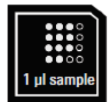
Loading the Ladder and the Samples

1 Pipette 1 µl of High Sensitivity DNA ladder (yellow ●) in the well marked .

2 In each of the 11 sample wells pipette 1 µl of sample (used wells) or 1 µl of marker (unused wells).

3 Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).

4 Run the chip in the Agilent 2100 Bioanalyzer within 5 min.

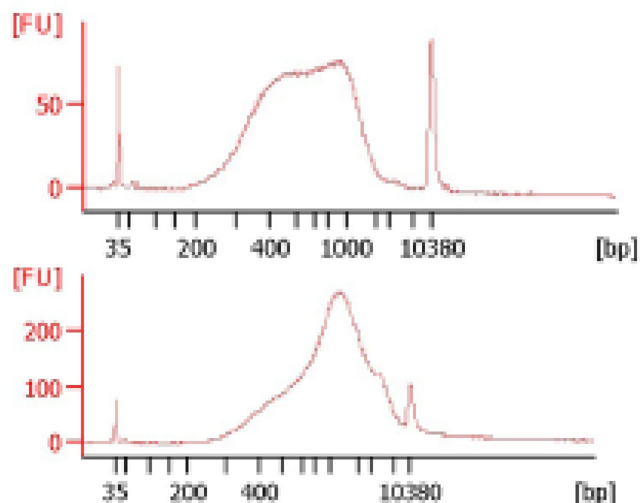


1) Place the vortexed chip in the Agilent 2100 Bioanalyzer.
2) In the open Agilent software, under “Contexts” (left pane of software) select “Instrument”
3) Click the “Assay” button in the upper right pane and choose “dsDNA High Sensitivity Assay” from the drop down.
4) Name the file in the “Destination and Data Acquisition Parameters” window (middle pane)
5) In the same window, select how many reactions or wells to run next to “Run sample” 1 to 11.
6) Identify sample names of each lane/well in the table at the bottom of the middle pane.
7) Click the “Run” button.
8) When the run is finished, remove the electrophoresis chip.

9) To clean the electrodes, put ~350µl of nuclease-free water in the electrode cleaner chip (clear plastic chip) on the machine and close the lid for 10 seconds and then dry the electrodes by opening the lid for 10 seconds.
10) Remove the electrode cleaner chip.

Data Analysis

- Most libraries made with the Illumina Nextera XT kit will have electropherograms that look similar to one of the two examples below (taken from the Illumina Nextera XT kit manual).



1) When the run is finished, select "Data" under "Contexts" (left pane of software) to see the electropherograms and virtual gel in one view.
2) Go to File, Print, and select the destination to save a PDF of the results.
3) With "Data" under "Contexts" selected, select on any run under "All Files". The resulting electropherograms of that run will populate the middle pane. Select an individual sample electropherogram and the selected electropherogram will enlarge to fill the middle pane.
4) There will be a table below the electropherogram. Below that table will be four tabs. Select the tab named "Region table".
5) There will be two blue vertical bars that will appear on the electropherogram.
6) With the mouse, position the two vertical bars on either side of the electropherogram peak (excluding the upper and lower marker peaks).

7) The Region table will now contain the average base pair length of your library within the two vertical bars.

Determining the Molarity of each Library

- Take the Qubit quantification (mass in ng/µl) and the Bioanalyzer-derived average fragment size in base pairs (size of fragment between the two vertical, blue bars) for each library and use the following equations to determine the molarity of each library (use a spreadsheet to calculate these numbers in a table format for ease):

1) (Average Base Pair Length x 607.8) + 157.9 = Molecular Weight

   Note: 607.8 is the average weight of all four bases. The 5' PO_4 weighs 157.9. Alternatively, the Illumina Nextera XT protocol recommends multiplying the average base pair length by a single value of 660 to get the MW.

2) Qubit (ng/µl) Concentration / Molecular Weight = Nanomoles/µl.
3) Nanomoles/µl x 1,000,000 = nM concentration.

Diluting and Pooling the Libraries

- The NCSU GSL requires pooled libraries to be at a minimum concentration of 10nM, while Genewiz requires a minimum concentration of 15nM.
- Dilute each library to the minimum concentration required by the NGS facility to which the libraries will be submitted for sequencing.
- Dilutions will produce the minimum concentration (we will say 15nM in this protocol) in 5µl.
- There are several ways to make dilutions, but the following protocol is how libraries have been diluted and pooled.

1) (5µl x 15nM) / (nM of library) = (volume to take from the library)
2) (5µl) – (volume to take from the library) = (volume of nuclease-free water) for the dilution
3) Sum all the "volumes of nuclease-free water" for each library that will be pooled. Pipet the summed value of nuclease free water into a 1.5mL microcentrifuge tube.
4) One by one, add each "volume to take from each library" to the nuclease free water in the 1.5mL microcentrifuge tube.
5) Vortex and spin down the tube.
6) Run 2µl of the diluted, pooled libraries on the Qubit as per the protocol above for the High Sensitivity dsDNA assay.
7) Run 1µl of the diluted, pooled libraries on a HS dsDNA Bioanalyzer chip as per the protocol above.
8) Using the Qubit concentration (ng/ul) and the Average Base Pair Length (bp), determine the molarity (nM) of the diluted, pooled library as per the protocol above which should be close to 15nM since each library was diluted to 15nM in the process.

9) Submit an aliquot of the pooled libraries as per the NGS company's requirements for volume and molarity.

II. Blue Pippin size-selected DNA

- Send at least 250ng of Total DNA in 30µl nuclease-free water to the GSL.
- Request size selection for a 0.8-3Kb target size range.
- Follow the protocol for Total DNA from leaf from infected cassava above except for adding 2µl of Blue Pippin size-selected DNA sample for each technical replicate into each RCA reaction.
- For sequencing from whiteflies, expect DNA concentrations to be very low after blue pippin size selection. Concentrations will increase after RCA.